Amendments to the Specification

Please amend the paragraph at page 5, lines 5-16, of the specification as follows:

Yet another type of assay system is termed "homogeneous assay." Homogenous assays can generally take place in solution, without a solid phase separation step, and commonly exploit chemical differences between the free probe and the analyte:probe complex. An example of an assay system which can be used in a homogenous or heterogeneous format is the hybridization protection assay ~~(HPA™)~~ (HPA) disclosed in Arnold, et al., U.S. Patent 5,283,174, in which a probe is linked to a chemiluminescent moiety, contacted with an analyte and then subjected to selective chemical degradation or a detectable change in stability under conditions which alter the chemiluminescent reagent bound or joined to unhybridized probe, without altering the chemiluminescent reagent bound or joined to an analyte:probe conjugate. Subsequent initiation of a chemiluminescent reaction causes the hybrid-associated label to emit light. This patent enjoys common ownership with the present application and is expressly incorporated by reference herein.

Please amend the paragraph at page 26, lines 17-27, of the specification as follows:

Yet another assay system having practical advantages in ease and rapidity of use may comprise an immobilized oligonucleotide having a portion complementary to a capturing oligonucleotide. The capturing oligonucleotide (capture probe) will contain a base sequence permitting hybridization to the target. The capturing oligonucleotide will also have a label attached within or near the target-binding nucleotide sequence region, such as a substituted or unsubstituted acridinium ester, which may be used in a homogeneous or semi-homogenous assay system to specifically detect hybrid nucleic acids without detecting single-stranded nucleic acids, such as the capture probe itself. Such a system favored by

Applicant is the ~~HPA™~~ HPA, which is discussed and incorporated by reference above. In the ~~HPA™~~ HPA format, the label contained on any capture probe which has not hybridized to its target will be hydrolyzed with the addition of base, while target:capture probe hybrid would protect the label associated therewith from hydrolysis.

Please amend the paragraph at page 37, line 18, through page 38, line 7, of the specification as follows:

Oligonucleotide probes of identical sequence containing varying amounts of 2'-O-methyl nucleotides were each individually hybridized to perfectly complementary synthetic RNA targets of the same length. The ~~probe sequence had SEQ ID NO:1~~ target sequence (SEQ ID NO: 1) and the ~~target sequence had~~ probe sequences (SEQ ID NO:~~2~~2-6). ~~The nucleotide base sequences of these oligonucleotides~~ were as follows (reading 5' to 3'):

~~SEQ ID NO:1 5'-GCTCGTTGCG GGACTT(AE)AACC CAACAT-3'~~

~~SEQ ID NO:2 5'-ATGTTGGGTT AAGTCCCGCA ACGAGC-3'~~

SEQ ID NO:1: atgttgggttaagtcccgcaacgagc;

SEQ ID NO:2: gctcgttgcgggacttaacccaacat (Probe A);

SEQ ID NO:3: **gcucguugcgggacuuaacccaacau** (Probe B);

SEQ ID NO:4: **gcucguugcggg**acttaacc**caacau** (Probe C);

SEQ ID NO:5: gctcgttgcggg**acuuaacc**caacat (Probe D); and

SEQ ID NO:6: gctcgttg**cgggacuuaacccaac**at (Probe E).

~~The~~ These probes~~, as illustrated below,~~ were synthesized to contain no 2'-O-methyl nucleotides (Probe A), all 2'-O-methyl nucleotides (Probe B), or a combination of deoxy- and 2'-O-methyl nucleotides

(Probes C, D and E), and each probe was labeled with an acridinium phenyl ester compound joined to a linker arm attached to the probe between nucleotides 16 and 17 (reading 5' to 3'). The bolded nucleotides represent 2'-O-methyl nucleotides. Probe C contained four contiguous deoxyribonucleotides positioned directly adjacent to each side of the linker attachment site and 2'-O-methyl ribonucleotides at all other bases; Probe D contained four contiguous 2'-O-methyl nucleotides positioned directly adjacent to each side of the linker attachment site and deoxyribonucleotides at all other bases, and Probe E contained eight contiguous 2'-O-methyl nucleotides positioned directly adjacent to each side of the linker attachment site and deoxyribonucleotides at all other bases. The T_m of each hybrid was determined using both a chemiluminescent and an optical method.

Please amend the paragraph at page 43, lines 11-18, of the specification as follows:

To compare the relative stabilities of hybrids containing various combinations of DNA, RNA, and 2'-O-methyl nucleotide strands, the following acridinium ester-labeled oligonucleotide probes ~~of SEQ ID NO:1 (see Example 1 above)~~ were hybridized to synthetic targets having a perfectly complementary base ~~sequence~~ sequences (reading 5' to 3'):

SEQ ID NO:2: gctcgttgcgggacttaacccaacat (DNA);
SEQ ID NO:3: gcucguugcgggacuuaacccaacau (2'-O-methyl nucleotides); and
SEQ ID NO:7: gcucguugcgggacuuaacccaacau (RNA).

As in Example 1, each probe was labeled with an acridinium phenyl ester compound joined to a linker arm attached to the probe between nucleotides 16 and 17 (reading 5' to 3'). The base sequences of the target sequences were as follows (reading 5' to 3'):

SEQ ID NO:1: atgttgggttaagtcccgcaacgagc (DNA);

SEQ ID NO:8: auguuggguuaagucccgcaacgagc (2'-O-methyl nucleotides); and

SEQ ID NO:9: auguuggguuaagucccgcaacgagc (RNA).

The probes and target sequences contained 100% ~~ribonucleotides (~~RNA~~)~~ (SEQ ID Nos. 7 and 9), 100% ~~deoxyribonucleotides (~~DNA~~)~~ (SEQ ID Nos. 1 and 2) or 100% 2'-O-methyl nucleotides (SEQ ID Nos. 3 and 8) in the combinations indicated in Table 5. The melting characteristics of each tested hybrid, as determined either using the chemiluminescent or the optical method, is shown in Table 5 below. More than one data point in the table indicates an independent, duplicate experiment.

Please amend the paragraph at page 44, lines 21-26, of the specification as follows:

~~An~~ The acridinium ester-labeled oligonucleotide probe of ~~SEQ ID NO: 1~~ SEQ ID NO:3 (see Example 1 above) ~~and composed of 100% 2'-O-methyl nucleotides~~ was allowed to hybridize to a completely complementary synthetic RNA target (SEQ ID NO:9) or DNA target (SEQ ID NO:1). Other than the fact that the oligonucleotide was labeled, hybridization and measurement of T_m were otherwise as described in Example 1 under the heading Optical Method. The results are shown in Table 5 above and are further illustrated in Figure 3.

Please amend the paragraph at page 45, lines 19-28, of the specification as follows:

a) In the first approach, 2 fmol of an acridinium ester-labeled probe having ~~SEQ ID NO: 1~~ the sequence of SEQ ID NO:2 or SEQ ID NO:3 (see Example 1 above) was hybridized to varying amounts of a completely complementary RNA target (SEQ ID NO:9) for a constant period of time, followed by differential hydrolysis and detection of the label. The hybridization was performed essentially as described

in Example 1, under the heading Chemiluminescent Method, with the following differences. Varying amounts of RNA target were allowed to hybridize with the labeled probe at 60°C for 45 minutes. Figure 4 shows the results of this experiment, wherein the probe ~~was either a DNA oligonucleotide~~ had either the DNA sequence of SEQ ID NO:2 (open boxes) or ~~consisted wholly of 2'-O-methyl nucleotides~~ the 2'-O-methyl nucleotide sequence of SEQ ID NO:3 (closed diamond); these results are also tabulated in Table 6 below. The degree of hybridization is expressed in Relative Light Units (rlu), which is a measure of the number of photons emitted by the acridinium ester label.

Please amend the paragraph at page 46, lines 16-22, of the specification as follows:

b) In a second approach, a constant amount (2 fmol) of the same target used in a) above was hybridized to varying amounts of the perfectly complementary probe for a fixed amount of time. Figure 5 shows the results of this experiment, wherein the probe ~~was either a DNA oligonucleotide~~ had either the DNA sequence of SEQ ID NO:2 (open boxes) or ~~consisted wholly of 2'-O-methyl nucleotides~~ the 2'-O-methyl nucleotide sequence of SEQ ID NO:3 (closed boxes). The hybridization and detection steps were the same as described in a) above, except that the hybridization reaction was carried out for 30 minutes rather than 45 minutes. The data are tabulated in Table 7 below.

Please amend the paragraph at page 47, lines 17-22, of the specification as follows:

c) As a third illustration of the ability of 2'-modified oligonucleotides to increase the rate of hybridization, fixed amounts of either modified or unmodified probe (1 fmol) and target (100 amol) were allowed to hybridize for varying amounts of time. The hybridization and detection protocols were otherwise the same as in b). Figure 6 shows the results, wherein the probe ~~was either a DNA oligonucleotide~~ had either the DNA sequence of SEQ ID NO:2 (open boxes) or ~~consisted wholly of 2'-O-methyl nucleotides~~

the 2'-O-methyl nucleotide sequence of SEQ ID NO:3 (closed diamonds). The data were as follows in Table 8 below:

Please amend the paragraph at page 48, lines 11-18, of the specification as follows:

d) The fourth method used to demonstrate the differences between the hybridization kinetics of 2'-modified and unmodified probes was a C_0t analysis. Acridinium ester-labeled probes of ~~SEQ ID NO:1~~ SEQ ID Nos. 2 and 3 (see Example 1 above) were used. Either a fixed amount of probe and varied amounts of target ("probe excess") or a fixed amount of target and varying amounts of probe ("target excess") were allowed to hybridize at 60°C for varying amounts of time. The fixed amount of either probe or target was 0.25 fmol and the variable amount of either probe or target included amounts in the range from 0.25 to 50 fmol. Hybridization was otherwise as indicated in Example 1, under the heading Chemiluminescent Method.

Please amend the paragraph at page 51, lines 21-27, of the specification as follows:

An acridinium ester-labeled oligonucleotide probe ~~of SEQ ID NO:1~~ having the sequence of SEQ ID NO:3 (see Example 1 above) ~~consisting entirely of 2'-O-methyl nucleotides~~ was allowed to hybridize to a perfectly complementary RNA target of the same length as described above. The hybridization and C_0t protocol were as described in Example 6(d), except that hybridization temperatures were either 60°C or 70°C. As shown by the data in Table 11 below, raising the temperature of hybridization of the 2'-O-methyl oligonucleotide to its target from 60°C or 70°C caused the hybridization kinetics to be accelerated 1.5 fold.

Please amend the paragraph at page 52, lines 10-17, of the specification as follows:

Hybridization kinetics are also accelerated by increases in salt concentration. The following example illustrates the effect of various concentrations of salt, e.g., LiCl, on the hybridization kinetics of 2'-O-methyl nucleotides. An acridinium ester-labeled probe ~~of SEQ ID NO:1~~ having the sequence of SEQ ID NO:3 (see Example 1 above)~~, consisting entirely of 2'-O-methyl nucleotides,~~ was allowed to hybridize, and a C_0t analysis conducted, as described in Example 6(d) above, at 80°C to an exactly complementary RNA target of the same length. Hybridization was performed at two different concentrations of LiCl. As shown in Table 12 below, increasing the salt concentration from 0.5 to 1.0 M LiCl enhanced the hybridization kinetics 2.9 fold.

Please amend the paragraph at page 53, lines 13-20, of the specification as follows:

To demonstrate the effect of simultaneously increasing the hybridization temperature and salt concentration on the hybridization kinetics of 2'-modified oligonucleotides, the following reactions were performed. An acridinium ester-labeled DNA oligonucleotide having the sequence of SEQ ID NO:2 and an acridinium ester-labeled oligonucleotide having ~~100% 2'-O-methyl nucleotides, both of SEQ ID NO:1~~ the sequence of SEQ ID NO:3 (see Example 1 above)~~;~~ were each separately allowed to hybridize to an exactly complementary RNA target molecule in a C_0t analysis. Hybridization conditions were as described in Example 6(d). Hybridization temperature and salt concentrations were as indicated in Table 13 below. The results were as follows:

Please amend the paragraph at page 54, lines 7-11, of the specification as follows:

The relative hybridization rates of labeled RNA, DNA and 2'-O-methyl-containing oligonucleotides to completely complementary DNA and RNA targets were individually determined. Rate determination was performed as disclosed in either Example 6(c) or 6(d) above. The labeled oligonucleotides had

~~identical base~~ the sequences of ~~SEQ ID NO:1~~ SEQ ID Nos. 2, 3 and 7 (see Example 1 above). The results are summarized in Table 14 below:

Please amend the paragraph at page 55, lines 9-13, of the specification as follows:

An RNA probe of ~~SEQ ID NO:1~~ SEQ ID NO:7 labeled with acridinium ester (see Example 1 above) and containing a non-nucleotide linker by which the label was attached to the probe was allowed to hybridize to an exactly complementary target which consisted entirely of either 2'-O-methyl or deoxyribonucleotides. Hybridization and C_0t analysis was done as Example 6(d). The results are expressed in Table 15 below.

Please amend the paragraph at page 56, lines 19-21, of the specification as follows:

Each helper probe was directed to rRNA base sequences close to the target site of one of the labeled probes. The degree of hybridization was measured using the hybridization protection assay ~~(HPA™)~~ HPA, as described above. The results are reported in relative light units (rlu).

Please amend the paragraph at page 60, lines 19-28, of the specification as follows:

The data presented in Example 13 indicates that 2'-O-methyl oligonucleotides hybridize to a detectable extent to RNA targets, even highly folded structures like rRNA, in the absence of helper probes. Nevertheless, helper probes can accelerate the hybridization of 2'-O-methyl oligonucleotides to highly structured RNA. To examine the effect of helper probes more closely, deoxy- and 2'-O-methyl oligonucleotide probes were hybridized to rRNA at different temperatures in the presence or absence of helper probes. Table 19 represents studies performed using acridinium ester-labeled probes having a

nucleotide sequence of Probe F of Example 2 above and helper probes c and d of Example 12 above. Table 20 represents studies performed using acridinium ester-labeled probes having ~~a nucleotide~~ the sequence of ~~SEQ ID NO:1~~ SEQ ID NO:3 (see Example 1 above) and helper probes g and h having 41 and 32 bases, respectively.

Please amend the paragraph bridging pages 61 and 62 of the specification as follows:

As a further demonstration of the effect of modified oligonucleotides on the performance characteristics of diagnostic probe molecules, a number of additional experiments were performed. These experiments were based on the Applicant's preferred detection method employing the ~~HPA™~~ HPA detection assay. In accordance with one ~~HPA™~~ HPA format, a chemiluminescent acridinium ester is attached to a probe and the probe is hybridized to an analyte. Following hybridization, chemiluminescence associated with unhybridized probe is selectively destroyed by brief hydrolysis in borate buffer. Since probe:analyte molecules are not destroyed in this process, the remaining chemiluminescence of hybridized probe is a direct measure of the analyte present. In this application, those acridinium ester-labeled probes which hydrolyze faster when unhybridized than when in a probe-analyte hybrid complex are preferred. Hydrolysis of probe and hybrid is pseudo first order and can be characterized by the value $t½$, which is the time, measured in minutes, required to hydrolyze 50% of the acridinium ester attached to either probe or hybrid. Thus, probes which exhibit a large differential hydrolysis (DH) ratio ($t½$ (hybrid)/$t½$(probe)) are highly desirable.

Please amend the paragraph bridging pages 63 and 64 of the specification as follows:

To examine whether modified nucleotides must be close to the site of label attachment to enhance the DH behavior of acridinium ester, ~~the~~ acridinium ester-labeled probes ~~of SEQ ID NO:1,~~ containing clusters of 2'-O-methyl nucleotides at different positions relative to the acridinium ester linker site~~;~~ were hybridized to a complementary RNA target. Labeled Probes A, B, C and D of Example 1 were used for this example. ~~Those nucleotides containing 2'-O-methyl substitutions are indicated below by underlining:~~

~~Probe M: 5'-GCTCGTTGCG GGACTT(AE)AACC CAACAT-3'~~

~~Probe N: 5'-GCTCGTTGCG GGACTT(AE)AACC CAACAT-3'~~

~~Probe O: 5'-GCTCGTTGCG GGACTT(AE)AACC CAACAT-3'~~

~~Probe P: 5'-GCTCGTTGCG GGACTT(AE)AACC CAACAT-3'~~

Please amend the table at page 64, lines 12-17, of the specification as follows:

Table 22

Probe	t½ (Probe)	t½ (Hybrid)	DH
~~M~~ A	.82, .8	48.7, 43.2	59.7, 54
~~N~~ B	.76, .6	90, 77.6	118.3, 129
~~O~~ C	.74	49.8	67.3
~~P~~ D	.44	81.4	185

Please amend the paragraph bridging pages 64 and 65 of the specification as follows:

As mentioned above, because of their higher thermal stability, the modified oligonucleotides of the

present invention are able to hybridize to a target nucleic acid at a higher temperature than unmodified oligonucleotides. At such higher temperatures, the hybridization rate, as well as the rate of other reactions, would be expected to increase. Among such other reactions is the rate of hydrolysis of acridinium ester labels. Because Applicant's preferred detection method employs the Hybridization Protection Assay ~~(HPA™)~~ HPA, described and incorporated by reference above, the following experiment was performed to determine whether benefits to the diagnostic assay conferred by an increase in hybridization rate would be offset by a decrease in the DH ratios of hybrid-associated and unassociated acridinium ester labels at this higher temperature.

Please amend the paragraph bridging pages 65 and 66 of the specification as follows:

The foregoing experiments were conducted using standard acridinium ester as the detectable chemiluminescent label. To examine whether the differential hydrolysis behavior of labels other than standard acridinium ester is enhanced by T_m-enhancing modified nucleotides, probes ~~of SEQ ID NO: 1 and~~ containing either ~~deoxy-~~ deoxyribonucleotides (SEQ ID NO:2) or 2'-O-methyl nucleotides (SEQ ID NO:3) were labeled in exactly the same manner and position (see Example 1 above) with standard acridinium ester, o-diBr acridinium ester, 2-Me acridinium ester, napthyl-acridinium ester, o-F acridinium ester, 2,7-diisopropylacridinium ester, or mixture of 1- and 3-Me acridinium ester, and their DH behavior examined. See Figure 1 for examples of acridinium esters. As summarized in Table 24 below, the use of modified nucleotide probes resulted in an increase in the DH ratio for all the acridinium ester derivatives tested by 1.1-6 fold.

Please amend the paragraph at page 67, lines 9-22, of the specification as follows:

To examine the relationship between hybridization kinetics and the number of 2'-O-methyl nucleotides within a probe sequence, ~~acridinium ester-labeled probes of SEQ ID NO:1 (see Example 1 above)~~ Probes A, B, D and E of Example 1 were synthesized, as well as the following probe sequences (reading 5' to 3') labeled in the same manner and location as the probes of Example 1 with an acridinium phenyl ester compound:. ~~These synthesized probes contained increasing amounts of 2'-O-methyl residues (underlined bases indicate the presence of 2'-O-methyl residues) on both sides of the AE linker:~~

~~Probe Q: 5'-GCTCGTTGCG GGACTT(AE)AACC CAACAT-3'~~

~~Probe R: 5'-GCTCGTTGCG GGACTT(AE)AACC CAACAT-3'~~

~~Probe S: 5'-GCTCGTTGCG GGACTT(AE)AACC CAACAT-3'~~

~~Probe T: 5'-GCTCGTTGCG GGACTT(AE)AACC CAACAT-3'~~

~~Probe U: 5'-GCTCGTTGCG GGACTT(AE)AACC CAACAT-3'~~

~~Probe V: 5'-GCTCGTTGCG GGACTT(AE)AACC CAACAT-3'~~

SEQ ID NO:10: gctcgttgcgggac**tta**acccaacat (Probe M); and

SEQ ID NO:11: gctcgttgcgggac**ttaa**cccaacat (Probe N).

The bolded nucleotides represent 2'-O-methyl nucleotides. The results are summarized in Table 25 below.

Please amend the paragraph at page 68, lines 6-17, of the specification as follows:

As summarized above, as few as 8 2'-O-methyl nucleotides (Probe ~~T~~ D) -- 4 on each side of the acridinium ester linker site -- were sufficient to accelerate the hybridization rate of an acridinium ester probe to the same level of a probe consisting almost entirely of 2'-O-methyl nucleotides (Probe ~~V~~ B). In contrast, the T_m of a probe containing four 2'-O-methyl nucleotides on each side of the acridinium ester linker site is lower than the T_m of a probe:target hybrid in which the probe contains additional 2'-O-methyl

nucleotides. Thus, according to the present invention it is possible to optimize, or "tune", the performance of an acridinium ester labeled probe with respect to its hybridization rate, differential hydrolysis, and melting properties. For example, an acridinium ester-labeled probe containing four 2'-O-methyl nucleotides on either side of the acridinium ester linker site will have its hybridization rate and differential hydrolysis properties maximally optimized, while a hybrid containing this probe will exhibit only a small increase in its melting temperature.

Please amend the paragraph at page 69, lines 8-17, of the specification as follows:

As a first consideration, probes having 22 bases and an acridinium-ester attached at a site located between a thymine base and a guanine base in Probes ~~W~~ Q and ~~Y~~ P and between two thymine bases in Probe ~~X~~ Q, but containing varying numbers of propyne-modified nucleotides, were hybridized to target rRNA in the presence of helper probes to examine the effect of the modification on the T_m of acridinium ester hybrids. Probe ~~W~~ Q contained no propyne modifications. Probe ~~X~~ Q contained two propyne modifications, one directly adjacent to each side of the label attachment site. Probe ~~Y~~ P contained 11 propyne modifications, including four contiguous modifications directly adjacent and 5' to the label attachment site and seven modifications located at bases spaced 3, 4, 6, 9-11 and 14 bases away from and 3' to the label attachment site.

Please amend table 26 at page 69, lines 23-27, as follows:

Table 26

Probe	Propyne Residues	T_m (chemiluminescent)	ΔT/Propyne
~~W~~ Q	0	71	---
~~X~~ P	2	72	0.5
~~Y~~ Q	11	82	1.0

Please amend the paragraph at page 70, lines 5-10, of the specification as follows:

To examine the effect of propyne groups on the hybridization kinetics of oligonucleotides, the rate of hybridization of the propyne-labeled probes of Example 20 to RNA were examined by C_0t analysis, as described in Example 6. As summarized below in Table 27, the probe containing two propyne groups (Probe ~~W~~ Q) hybridized at the same rate as the probe containing no propyne groups (Probe ~~X~~ P), while the probe containing 11 propyne groups (Probe ~~Y~~ Q) hybridized 1.9-fold faster.

Please amend the table at page 70, lines 12-16, as follows:

Table 27

Probe	$C_0t_{½}$	Relative Rate
~~W~~ Q	0.75×10^{-5}	1
~~X~~ P	0.81×10^{-5}	0.93
~~Y~~ Q	0.39×10^{-5}	1.9